Does pain at an earlier stage of chondropathy protect female mice from structural progression after surgically induced osteoarthritis?

I S von Loga[1], V Batchelor[1], C Driscoll[1], A Burleigh[1], S-L Chia[1], B Stott[1], J Miotla-Zarebska[1], , D Riley[1], F Dell'Accio[2], T L Vincent*[1]

[1]Kennedy Institute of Rheumatology, University of Oxford. [2]William Harvey Institute, Queen Mary University of London.

Authors:

Isabell S von Loga, PhD

Vicky Batchelor, BSc (hons.)

Clare Driscoll, PhD

Annika Burleigh, PhD

Shi-Lu Chia, MD, PhD

Bryony Stott, PhD

Jadwiga Miotla-Zarebska, PhD

David Riley,MRes

Francesco Dell'Accio, MD, PhD

Tonia L Vincent, MD, PhD

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*** Corresponding author:**

Prof Tonia Vincent

Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Oxford OX3 7FY

01865 612645, tonia.vincent@kennedy.ox.ac.uk

Conflict of interest

TLV ad hoc consultancy in past 2 years for Mundipharma and GSK. FDA has received consultancy fees from UCB and Samumed.

Abstract

Objective: Female C57BL6 mice exhibit less severe chondropathy compared with male mice. This paper tests the robustness of this observation and explores potential underlying mechanisms.

Methods: OA was induced in male and female C57BL6 or DBA1 mice (n=6-12 per group) using destabilisation of the medial meniscus (DMM), or partial meniscectomy (PMX). Some mice were ovariectomised (OVX) (n=30). *In vivo* repair was assessed by focal cartilage defect or by joint immobilisation (sciatic neurectomy) after DMM. Histology, gene expression of whole knees, behavioural analysis using Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS), and by Linton incapacitance, were used (n=7-10/group).

Results: Female mice displayed less severe chondropathy (20-75% reduction) across both strains, and after both surgeries. Activity levels 10 weeks after PMX were similar between males and females. Some repair-associated genes were increased in female joints after surgery but *in vivo*, no repair differences were evident. Despite having reduced chondropathy, females developed pain-like behaviour at the same time as male animals. At established pain-like behaviour (10 weeks post PMX), females differentially upregulated pain-associated genes including glial cell derived neurotrophic factor, neurturin, and neurotrophic factors 3 and 5. Inflammatory genes were not regulated in painful joints in either sex.

Conclusion: We confirm strong structural joint protection in female mice which is not due to activity or intrinsic repair differences. Female mice develop pain at the same time as males,when a distinct set of neurotrophins are induced. We speculate that heightened pain sensitivity in females protects the joint by preventing overuse.

Introduction

Epidemiological studies identify female sex as a major non-modifiable risk factor in OA development[1]. Sex-dependent associated risk holds true across different joint sites and at different ages and is particularly apparent after 55 years of age[2-4]. In the hand, the sex bias is particularly striking; 90% of those attending secondary care are female and the incidence peaks around the perimenopausal period[1,3]. The burden of hand OA in women increases as those individuals age[2] tending to plateau at around the age of 80 (as does male disease)[3,5]. Males have significantly higher cartilage volume at all sites compared with females, even after accounting for differences in height, bone size and weight. This difference becomes more apparent after the age of 50[6]. The sex bias in OA incidence and prevalence, particularly after 55 years of age, suggests an association with sex hormones and their changes at the time of the menopause. Despite the speculation that hormone replacement could protect female joints from OA, a systematic review of epidemiological studies has failed to support a joint-protective role of oestrogen in OA[7].

OA risk is also sex-dependent in mice. Young female mice subjected to destabilisation of the medial meniscus (DMM) develop less severe OA than male mice[8]. In this study, female mice that had been ovariectomised (OVX) (at 6 weeks of age) had significantly more severe OA than non-ovariectomised female mice. Conversely, orchidectomy (castration) in male mice reduced disease severity compared with non-operated males[3,8]. Similar studies have been performed in a number of different species[3, 9], and a systematic review comparing the severity of OA after OVX concluded that OVX led to worse disease compared with non-ovariectomised females in around two thirds of cases[10]. The effects of exogenous oestrogen treatment are less conclusive. Only half of the studies looking at the effects of oestrogen replacement found reduced OA severity, and one study found exacerbation of disease[10]. Lack of conclusive results in these studies may be due to a failure to appreciate the complexity of the effects of multiple hormones affecting joint tissues, as well as inconsistency in study design and power. In animals as well as humans, ovariectomy affects the expression of several hormones besides oestrogen including decreases in progesterone and testosterone, and increases in follicle-stimulating hormone (FSH) and luteinising hormone (LH)[10]. Receptors for oestrogen, androgens and FSH have been described in joint cells, including articular chondrocytes, and have been shown to affect tissue metabolism[10-12].

If one regards OA as a disease of failed tissue repair, information may be gleaned from sex-dependent wound responses in other tissues. Several other tissues exhibit sex differences in their response to injury. One that has been extensively examined is cutaneous wound healing. Ageing of healthy human skin is associated with reduced levels of transforming growth factor beta (TGFß)-1, reduced rate of cutaneous wound healing, albeit with reduced scarring. This age-related pattern can be reversed by systemic oestrogen therapy[13]. Similar relationships can be demonstrated in mice. Young female mice have significantly higher levels of Tgfß-1 than young males, and exhibit an increased inflammatory response to injury[13]. Ovariectomised females demonstrate delayed repair of incisional wounds which can be reversed by topical application of oestrogen, which was associated with increased latent Tgfß-1 secretion by dermal fibroblasts[13]. Orchidectomised mice display accelerated wound healing compared with non-castrated controls[14].

There are also sex differences in the perception of pain. Females patients present with more chronic pain conditions than males, and are known to experience higher levels of pain and have a lower threshold for pain[15-18]. The actual mechanisms of these sex differences is as yet unclear[17]. Females have been suggested to employ different immune cells in their responses to pain[19,20]. For example, in mice, females but not males, used adaptive immune cells within the spinal cord in the development of mechanical hypersensitivity[21].

In this paper we explore sex differences in murine OA in detail with a particular focus on the mechanisms that may drive differences in disease severity. We examine the impact on activity levels, ability to repair cartilage, molecular response to injury, and pain-like behaviour.

Methods

Animals and surgical methods are described in [22,23] with further details presented in Supplementary Methods.

Sciatic Neurectomy: Animals were placed under general anaesthesia by inhalation of isoflurane (Vetpharma, Leeds, UK); 3% induction, 1.5-2% maintenance in 1.5-2 L/min O2. Hindlimbs were shaved and prepared for aseptic surgery. 0.3 mg/ml buprenorphine (Vetergesic Alstoe Animal Health, UK) was administered subcutaneously to all animals for analgesic purposes. The hindlimbs were stretched out and secured using surgical tape. From the midline, around 0.5cm lateral to the tail, a

1cm incision was made and the fascia and hind leg muscles separated. The sciatic nerve was exposed, lifted from the limb and a 2-4mm segment of the nerve was removed, causing minimal damage to the surrounding muscle, before the skin was closed and sutured.

Focal Cartilage Injury: 10 week old male and female C57BL6 mice underwent focal cartilage injury in the patellar groove as previously described[24]. 8 weeks after injury, joints were scored using the previously described modified Pineda score[25] to assess intrinsic cartilage repair.

Histology: Mice were sacrificed by CO_2 inhalation and the ipsilateral knee joints were collected for histological analyses by sharp division at the proximal femur and distal tibia. The skin and surrounding tissue were removed and joints subsequently fixed in 10% formalin for 24 hours before decalcification in 20% formic acid for one week. The tissue was paraffin-embedded and coronal sections of 4 microns were cut at 80 micron intervals. Tissue slides were stained using Safranin-O and in the case of the PMX histology also FastGreen for microscopic inspection and histological scoring. Histological analysis of the knees was done by blinded scoring by two observers following the Osteoarthritis Research Society International (OARSI) scoring system[26]. Each joint consisted of eight to twelve scored sections. Each quadrant surface (lateral femoral condyle and tibial plateau and medial femoral condyle and tibial plateau) within the joint was scored separately, and the score from each histological section was added up to provide a section score. The three highest section scores obtained for any given joint were summed to yield a cartilage damage index for that animal.

Osteophyte scoring: This was assessed either by histological grading of size and maturity according to previously validated methods[27] or by micro-CT (ipsilateral and contralateral joints) as previously described by our group[28].

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR): RNA was extracted from whole knee joints, as described previously[29]. Briefly, after skin and muscle were removed the joint was trimmed to the upper and lower edges of the parapatellar and quadriceps tendon then snap frozen in liquid nitrogen. Whole knees were freeze-fractured using a pre-cooled bio-pulveriser. RNA from the powdered sample was isolated using RNeasy RNA mini kits (RNeasy mini kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions, then stored at -80°C. Samples were

reverse transcribed into cDNA using a high capacity RNA to cDNA kit (Applied Biosystems, CA, USA). Murine cDNA was analysed on two custom-made microfluidic cards (Taqman, Thermo Scientific) including hydrolysis probes, testing a total of 67 OA-related or pain-regulating genes (see Supplementary Table 1). Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, where each gene of interest was compared to a housekeeper gene (either Fibroblast growth factor receptor 3, *Fgfr3,* or Glyceraldehyde 3-phosphate dehydrogenase, *Gapdh)* and normalised to the respective control group (naïve or sham) for each sex.

Weight bearing: Static weight bearing measurements were performed using the Linton Incapacitance Tester (Linton Instrumentation, Norfolk, UK), as previously described[30, 31]. Briefly, mice were first acclimatised to the chamber on two separate occasions over two weeks before experimental measurements. Mice were manoeuvred inside the chamber to stand with one hindlimb on each weighing scale. The weight placed on each hindlimb was measured over a one second interval for at least three consecutive measurements. Results were expressed as the percentage of weight transmitted through the operated compared with the contralateral limb. One female observer each (I.v.L. for PMX, C.D. for DMM experiments) performed the measurements and was blinded to the treatment status of the mice.

LABORAS: Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS) allows for the measurement of mouse activity in an undisturbed environment overnight. Mice were housed singly, with 4 cages in use at any one time. They were given food and water *ad libitum*. Cages contained sawdust bedding but no additional enrichment. LABORAS picked up vibrations of animal movement and converted these into behaviour classifications, specifically "climbing", "locomotion", "inactivity", "rearing", "grooming", "drinking", and "eating". Measurements were performed during the mouse's 12h active period (excluding the initial exploratory phase) 19:00-07:00. Total duration for each activity over the 12 hours was calculated.

Statistical analyses: Data are expressed as mean ± standard error of the mean (SEM) and were analysed using GraphPad Prism (GraphPad Software, San Diego, CA). For behavioural comparisons of two groups only a t-test with Bonferroni multiple comparisons test, where applicable, was used. For behavioural comparisons of three groups and without a repeated-measures timecourse, an ordinary

two-way ANOVA with Tukey multiple comparisons test was applied. For any timecourse experiments, a repeated-measures two-way ANOVA with a Bonferroni test for multiple comparisons was used. In case of missing timepoints, a mixed model with a Bonferroni multiple comparisons test was used instead of a repeated-measures two-way ANOVA.

Results

Do female mice develop lower chondropathy scores after surgical joint destabilisation?

We examined sex differences in chondropathy score after joint destabilisation in a number of different groups, specifically asking whether sex differences were robust (1) after different types of surgery (DMM or PMX), and (2) across different genetic strains (C57BL6 or DBA). Lower disease severity in female mice was apparent following both DMM and PMX surgery from 8 weeks (DMM) and 12 weeks (PMX) (Figure 1A, B, C). Following PMX, disease severity plateaued in both female and male mice 12 weeks after PMX. OVX females did not have different disease scores following PMX compared with female controls at any timepoint examined. In another strain, female DBA1 mice also displayed reduced chondropathy after DMM compared with males (Figure 1D). Osteophyte volume and maturity was also assessed (Figure 1E, F). This was measured by histology and by micro-CT, using the epiphyseal volume as a surrogate marker for osteophyte size according to our validated method[28]. Osteophyte maturity was similar between male and female mice. Osteophytes were smaller in female mice than males, but as a proportion of the total volume of the epiphysis, this represented the same percentage increase (Figure 1G, H).

Are female mice less active than male mice?

One possible explanation for the decreased disease observed in female mice was reduced joint use through reduced activity. We assessed activity levels of males and females using LABORAS over a 12-hour period. LABORAS allows the recording of different behavioural activities of single-caged animals. It is able to distinguish between ‘climbing’, ‘locomotion’, ‘immobility’, ‘grooming’, ‘drinking’, and ‘eating’. When comparing the mean activity over the 12h period 10 weeks after PMX, there were no significant differences between sham and PMX groups for either sex, and no apparent differences between sexes. This was the case when considering the total activity over 12 hours or after breaking activity levels down to one-hour periods (data not shown). (Figure 2). Similar activity levels were seen

in naïve (non-surgery) male and female mice (data not shown). The full male data set will be published elsewhere (manuscript in revision). These data indicate that LABORAS is not a sensitive tool for detecting pain-like behaviour, but do not support that differences in activity levels account for differences in structural joint damage.

Do female mice repair cartilage better than male mice?

We next tested the hypothesis that female mice are more able to repair damaged cartilage than male mice and this could account for reduced chondropathy scores. We first assessed gene expression in response to acute joint destabilisation by extracting RNA from the whole knee joint 6 hours after DMM. The analysis focused on pathways implicated in cartilage repair including fibroblast growth factors (FGF) (FGF2, FGF18, FGFR1-2,4) and TGFß (TGFß1-3, TGFßR2-3). Pmepa1, Bmpr2 and Ltbp2 had previously been shown by our group to be strong TGFß-dependent genes in human chondrocytes[33] (Table 1). Several genes were only upregulated in female joints and three were significantly higher in female compared with male mice: Bmpr2, Fgf2, and Pmepa1. Another TGFß family member that is strongly induced by FGF2, Inhibin A (Inhba) (the homodimer of which forms activin A) was upregulated equally in both male (5.92 ± 1.14, $p<0.01$) and female joints (6.33 ± 0.60, $p<0.0001$).

Gene expression data suggested that the response to joint injury may produce a stronger anabolic response in female compared with male mice. We investigated whether this related to improved repair of articular cartilage after injury *in vivo*. Previously published data from our lab showed that OA development could be prevented by performing joint immobilisation by sciatic neurectomy at the time of joint destabilisation[29]. Others have previously demonstrated that joint immobilization facilitated joint repair after injury[34, 35]. To test whether female mice exhibited enhanced cartilage repair *in vivo*, we used two different models. In the first we used a focal cartilage defect model originally described by Eltawil and colleagues[24]. In this model, which had historically been tested in male mice only, the authors showed both an age and strain dependent influence on repair with only young DBA1 mice exhibiting repair of the focal defect[24].

Male and female C57BL6 mice underwent focal cartilage injury at 10 weeks of age in order to see whether females were more likely than males to repair their cartilage. Joints were examined 8 weeks later. Cartilage repair was assessed by a modified Pineda score[25] (Figure 3A, B). No differences in repair were observed between the two groups. In a second model, we assessed whether joint

immobilisation by sciatic neurectomy, after disease was established, would allow reversal (repair) of cartilage damage induced by DMM surgery. This model has not been previously validated and we selected different timepoints for both male and female mice to ensure that there was a sufficient level of chondropathy at the start of the experiment. In both male and female mice neurectomy was able to arrest disease progression, but did not reverse disease (Figure 3C, D). Collectively, these data suggest that despite having a more "repair-conducive" molecular response to joint destabilisation, male and female C57BL6 mice both fail to exhibit functional repair *in vivo*.

Do male mice mount a more catabolic response upon joint destabilisation?

As the inflammatory response to injury has been shown to be sex-dependent in other tissues, we next addressed whether there was a difference in inflammatory gene regulation between sexes that could account for a more catabolic response in male joints. We assessed genes associated with the inflammatory response in both males and females 6 hours after DMM in the whole knee joint compared with naive mice of each sex (Table 1). Inflammatory genes were upregulated to a similar extent in both male and female joints. MMP-19 was the only gene that was significantly different between sexes (higher in females than males; 4.45 ± 0.56, 2.46 ± 0.29 respectively) ($p<0.05$). Based on these early 6-hour results, we concluded that differences in the inflammatory response to injury was unlikely to explain the sex differences seen in chondropathy.

Do male and female mice display different OA pain-like behaviour?

We next tested whether females displayed different pain-like behaviours to male mice. We assessed spontaneous pain-like behaviour after sham or OA surgery (DMM or PMX) in male, female, and OVX (PMX only) mice using Linton incapacitance testing, which measures weight asymmetry through the hind limbs. Spontaneous pain-like behaviour was seen transiently in the post-operative period in all groups and then from around 9 weeks after PMX and 10 weeks after DMM (Figure 4A, B). No difference in the timing or severity could be discerned between the sexes. These results were somewhat surprising as we had previously concluded that chondropathy score predicted time of onset of late OA pain-like behaviour in male mice[36,37] through release of pain sensitising molecules such as nerve growth factor (NGF) from the damaged articular cartilage[36]. These results suggested that females either have an exaggerated behavioural response to modest cartilage damage or that

additional pain sensitising pathways are activated, perhaps elsewhere in the joint, that were independent of cartilage loss. To test these hypotheses further, we assessed the differences in joint molecular profile at the time of established OA pain-like behaviour (10 weeks after PMX).

Is the molecular pain response different between male and female mice?

To assess sex differences in the regulation of genes associated with pain, we assessed a panel of pain-related genes that we had previously used as a screening tool at a time of established late OA pain[36]. This list included 29 genes that were either associated with inflammation (cytokines, chemokines, and leukocyte activation markers) or were known pain sensitisers. We compared the expression of these molecules in whole knee joints between sexes, 10 weeks after PMX surgery (when pain-like behaviour was established) (Table 2). No statistically significant regulation in inflammatory genes was seen in either group, in keeping with our previously published data, and suggesting that inflammatory changes in the joint are not a major driver of pain-like behaviour at this time[36]. *Il15* and *Nos*2 were relatively higher in females (*Il15* 1.51 ± 0.23, *Nos2* 1.32 ± 0.14) when compared with males (*Il15* 0.75 ± 0.13, *Nos2* 0.85 ± 0.09, $p<0.05$) although neither were upregulated significantly above the respective sham control. In contrast, when considering the pain sensitising molecules, significant sex-dependent changes were observed. Male mice showed significant increases in *Ngf* (1.76 ± 0.14, $p<0.01$) and *Bdkrb1* (2.48 ± 0.34, $p<0.05$) in keeping with our previous publication[36]. Females, had strong and significant upregulation of a group of neurotrophins including *Gdnf* (2.54 ± 0.30) and *Nrtn* (6.71 ± 1.24) when compared with their respective sham or male PMX control. A further two neurotrophins *Ntf3* (1.92 ± 0.27), and *Ntf5* (2.89 ± 0.48) were upregulated compared with males ($p<0.05$, $p<0.001$ respectively), but not significantly compared with their sham control. *Pspn* was downregulated in females (0.40 ± 0.12) compared with males (1.19 ± 0.27, $p<0.05$). Raw CT values are shown for both the inflammatory and pain-related molecules in the sham operated and OA groups in Supplementary Table 2. No significant differences between sexes in sham basal activity in any of these genes was detected (by t test, data not shown). Collectively these results suggest that females develop pain-like behaviour after less severe cartilage damage and may use different molecular pathways to drive pain-like behaviour.

Discussion

We have confirmed a previous report showing that male mice develop more severe chondropathy after surgical joint destabilisation compared with females[8] and extended the previous study by showing that this was robust across different strains and surgical induction methods. We were unable to confirm a reversal of female protection in OVX animals. This is not easily explained but it is the case that several other studies have failed to observe this also[10]. OVX mice were purchased from a commercial provider and we were able to verify oestrogen deficiency by oestrogen dependent gene expression (data not shown). The previously published Ma et al. study concluded by suggesting that lower chondropathy might be due to different activity levels between males and females (higher activity predisposing to increased disease). We were unable to substantiate this using LABORAS. We did not find evidence of different activity levels between the two sexes, although we were also unable to distinguish activity levels between PMX and sham operated animals in either sex indicating that LABORAS is not very sensitive. Further details of this insensitivity will be published elsewhere (manuscript in revision).

Next, we explored sex differences in the molecular response to joint destabilisation, addressing specific clusters of disease modifying genes. Looking at the immediate post injury gene expression profile in whole knee joints, we saw no difference in the inflammatory/catabolic genes between male and female mice but did see some differences in genes relating to the TGFß pathway, suggesting that females could elicit an enhanced repair response after injury. This was subsequently tested in two models of *in vivo* repair. No differences between the sexes were observed and we concluded that their repair profile (or lack thereof), at least in these stringent models, was similar.

Interestingly, we found the same time of onset and severity of pain-like behaviour in male and female mice following either DMM or PMX. This has recently been confirmed by another group following the medial meniscal transection model (MMT) in which the authors also confirm reduced chondropathy in female mice[38]. This was a little surprising in view of the fact we had previously suggested that the onset of pain-like behaviour in mice arises at the time that the junction between the calcified and non-calcified cartilage (tidemark) is breached[36]. These results could have suggested that pain-like behaviour in female mice was originating from a tissue other than the cartilage, such as the synovium. Although we can't completely exclude this possibility, the inflammatory gene profile in the joint at the onset of pain-like behaviour indicated that there was little joint inflammation present in either male or female OA joints suggesting that synovitis was unlikely to be contributing to the pain

phenotype. This suggests that female OA pain-like behaviour in this model is 'non-inflammatory' as we have concluded previously in males[36]. We also studied osteophyte development in male and female joints. Osteophyte development in murine OA occurs rapidly after joint destabilisation (within the first two weeks)[28] and does not correlate temporally with pain-like behaviour changes. No differences in the size (by % volume of the ephiphysis) or maturity of the osteophyte were detected between male and female mice.

At the time of established OA pain-like behaviour, it appears that female mice regulate a different, additional set of potential pain-modulating genes. These included *Gdnf* , *Nrtn* , *Ntf3 , Ntf5* and *Pspn* . GDNF, NRTN and PSPN are trophic factors for dopaminergic motoneurons and midbrain neurons and are being explored in Parkinson's disease[39,40]. They signal through a common receptor family and are all part of the TGFβ superfamily[41]. GDNF and NRTN and have been associated with inflammatory bone pain[42], and GDNF has also been suggested as an analgesic target in neuropathic pain[39,40]. As we found that TGFß activity was increased in females after joint injury compared with males, it is possible that a common mechanism exists for the activation of TGFβ superfamily molecules. NTF3 and NTF5 are both neurotrophic factors related to NGF. NTF3 and NTF5 are largely regarded as a survival factors for sensory neurons[43,44], but NTF3 has also been shown to have analgesic effects specifically by down-regulating TRPV1 and suppressing thermal hyperalgesia[45]. No sex differences have been reported for the regulation of any of these molecules. NGF, a well-known pain target for OA[46,47], was not differentially regulated between the sexes, suggesting that it contributes to OA pain-like behaviour in both male and female mice, although in this analysis NGF was not significantly increased in female joints above sham-operated joints. It would have been nice to check that both male and female mice responded equally to anti-NGF therapy, but this was not available to us.

Our data are consistent with mounting evidence that shows pain can be relayed by different mechanisms in males and females, suggesting that there are common as well as distinct sex-specific pathways[48]. Females have been shown to employ cells of the adaptive immune system to relay pain-like behaviour[21]. Although we can't completely exclude a mechanism that involves adaptive immune cells in the present study, we did not see any evidence of classical inflammatory gene regulation in either male or female joints at the time the mice developed pain-like behaviour.

318 The inclusion of women in clinical studies has only become mandatory in the US in 1993,
319 whilst preclinical studies still do not require the inclusion of both sexes[17,49]. Part of this is due to fear of
320 oestrous cycle-related variability in females, although it has been suggested that fighting to establish
321 dominance hierarchies in male mice can introduce as much variability, if not more[17]. The inclusion of
322 females in preclinical studies is important to identify female-specific pathways in drug discovery and to
323 avoid results from male animals being falsely generalised to females[17,49]. The marked female
324 predominance in chronic pain conditions; higher documented pain sensitivity, lower pain thresholds,
325 and higher pain ratings[15-18] reinforces the fact that preclinical studies probably need to be done in both
326 sexes if we are to attempt to understand their molecular basis. This approach is already being
327 implemented by the NIH in the US[50]. This work has important implications for NC3Rs. Whilst the goals
328 of replacement, reduction, and refinement must be upheld, it may be necessary to study such
329 pathways in both sexes in order not to miss sex-specific differences. What is also evident is that it
330 would not be appropriate to perform analyses of mixed populations of male and female animals in
331 mechanistic studies.
332 Finally, could chondroprotection in female mice be as a result of earlier pain sensitisation?
333 Given the highly mechanosensitive nature of OA[29], we speculate, as others have done previously,
334 that pain protects the joint by preventing overuse. As female mice are sensitized at an earlier stage of
335 their disease this could be one mechanism to explain the reduced chondropathy observed. This
336 debate is topical in view of the discussion around rapidly progressive OA in individuals receiving good
337 analgesic control with therapies such as anti-NGF[51].

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Figure Legends:

Figure 1: Reduced chondropathy scores in female mice following joint destabilisation. OARSI scores of ipsilateral joints after DMM surgery in male and female C57BL6 mice (A). OARSI scores after PMX in male, female and OVX female mice (B). The male data of weeks 8 and 12 has previously been published as a supplementary figure[32]. Representative histological sections of the medial compartment of the joint (C) from histological scores in (B). OARSI scores after DMM surgery in male and female DBA1 mice (D). Osteophyte size (E) or maturity (F) by histology. Osteophyte size by micro-CT shown as total epiphyseal volume of right (R) (operated) and left (L) (non-operated) epiphyses (G), or expressed as the % increase over the unoperated side (H). SEM shown. (A, B, D) Two-way ANOVA or, in the absence of the same sample size within a timepoint, mixed effect analysis with Bonferroni post hoc for multiple testing. Asterisks indicate un-paired t-test (E-F), and paired t-test (G-H). Only significant differences shown *p<0.05, ***p<0.001, ****p<0.0001 comparing male with females in all cases except in (G).

Figure 2: Activity differences in male and female C57BL6 mice 10 weeks after PMX or sham surgery. Male and female mice, 10 weeks after PMX surgery, were singly housed in LABORAS recording cages for 18h overnight (15:00 - 9:00) (n=7-10/group) with analysis performed between 19:00 and 07:00. No significant differences were observed between PMX and sham groups in either males or females. Activity levels were similar between sexes. One-way ANOVA with Bonferroni post hoc for multiple testing. SEM shown.

Figure 3: Female mice do not exhibit enhanced cartilage repair compared with male mice. Cartilage repair scores 8 weeks after focal cartilage injury of the intercondylar groove (A) with representative images at 0 and 8 weeks after injury (B). No significant differences were observed between groups. OARSI scores of joints of male mice following either DMM alone or DMM followed by joint immobilisation (by sciatic neurectomy, ScN) at either 4 or 8 weeks post DMM (C). OARSI scores of joints of female mice following DMM alone, or DMM followed by joint immobilisation (by sciatic neurectomy, ScN) at 12 weeks (D). For both males and females, Joint immobilisation halted disease progression. Ordinary one-way ANOVA with Tukey post hoc for multiple comparisons. SEM shown.

Figure 4: Male and female mice exhibit similar pain-like behaviours after either DMM or PMX. Pain-like behaviour was tested in male and female mice by Linton incapacitance at a variety of times after surgical induction of OA by DMM (A) or PMX (B). Significant differences shown compared with sham group by mixed effects analysis with a Dunnett post hoc for multiple comparisons (A) or repeated measures two-way ANOVA with Sidak post hoc for multiple comparisons (B). SEM shown. n=10 DMM, n=15 PMX, n=6 Shams, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table 1: Average fold changes (FC) of expression of genes associated with repair and inflammation in the whole knee joints 6 hours after DMM surgery normalised and expressed relative to the respective naive control. Asterisks indicate unpaired t-tests within each sex, p-values compare 6-hour data across sexes by unpaired t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). SEM shown.

Table 2: Average fold changes (FC) of expression of genes associated with inflammation and pain in the whole knee joints 10 weeks after PMX surgery normalised and expressed relative to the respective sham-operated group. Asterisks indicate unpaired t-tests within each sex, p-values compare 10-week PMX data across sexes by unpaired t-test (*p<0.05, **p<0.01,). SEM shown.

Supplementary Table 1: Genes examined on TaqMan Low-Density Array (TLDA) microfluidic cards

Supplementary Table 2: Average raw CT values (± SEM) of genes associated with inflammation and pain in whole knees 10 weeks after PMX or sham surgery

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	Naive Male (normalised)	6h DMM Male FC±SD	Naive Female (normalised)	6h DMM Female FC±SD	Mal 6h D
Repair					
	1.02 ± 0.10	1.04 ± 0.09	1.00 ± 0.01	1.52 ± 0.13**	p<0.
	1.03 ± 0.16	1.68 ± 0.17	1.01 ± 0.09	2.11 ± 0.39*	
	1.04 ± 0.16	1.61 ± 0.21	1.00 ± 0.06	1.44 ± 0.07**	
	1.04 ± 0.16	1.52 ± 0.32	1.01 ± 0.09	2.53 ± 0.03****	p<0.
	1.04 ± 0.18	1.05 ± 0.16	1.02 ± 0.12	1.30 ± 0.10	
	1.02 ± 0.13	0.95 ± 0.08	1.01 ± 0.07	0.87 ± 0.03	
	1.04 ± 0.14	0.64 ± 0.08	1.05 ± 0.18	1.41 ± 0.32	
	1.02 ± 0.12	5.92 ± 1.14**	1.01 ± 0.06	6.33 ± 0.60****	
	1.01 ± 0.09	1.05 ± 0.23	1.01 ± 0.10	0.97 ± 0.07	
	1.00 ± 0.05	0.97 ± 0.13	1.01 ± 0.09	1.25 ± 0.08	
1	1.03 ± 0.16	1.37 ± 0.22	1.01 ± 0.09	2.46 ± 0.27***	p<0.
	1.03 ± 0.17	1.07 ± 0.10	1.02 ± 0.12	1.17 ± 0.08	
	1.02 ± 0.11	0.69 ± 0.08	1.01 ± 0.09	0.73 ± 0.05	
	1.00 ± 0.06	0.72 ± 0.06	1.02 ± 0.14	1.00 ± 0.26	
	1.02 ± 0.10	1.20 ± 0.20	1.00 ± 0.05	1.20 ± 0.06	
	1.07 ± 0.21	1.03 ± 0.10	1.02 ± 0.13	1.04 ± 0.07	
	1.03 ± 0.16	0.86 ± 0.19	1.02 ± 0.12	1.30 ± 0.15	
ammatory					
;1	1.03 ± 0.17	4.52 ± 1.06**	1.01 ± 0.07	6.65 ± 0.71****	
;4	1.08 ± 0.24	6.75 ± 2.52*	1.01 ± 0.09	12.05 ± 1.11*	
;5	1.05 ± 0.19	1.92 ± 0.55	1.01 ± 0.06	2.04 ± 0.20	
	1.11 ± 0.31	61.05 ± 24.71	7.35 ± 7.02	44.75 ± 6.55***	
	1.04 ± 0.18	1.03 ± 0.16	1.00 ± 0.05	1.67 ± 0.25	
	1.05 ± 0.19	1.17 ± 0.15	1.02 ± 0.12	1.13 ± 0.02	
	1.14 ± 0.33	0.95 ± 0.23	1.07 ± 0.24	1.08 ± 0.22	
	1.04 ± 0.16	6.29 ± 1.23**	1.01 ± 0.06	6.31 ± 0.40****	
	1.20 ± 0.38	131.49 ± 49.15	1.00 ± 0.05	129.19 ± 24.35***	
3	1.05 ± 0.20	1.30 ± 0.23	1.01 ± 0.07	1.63 ± 0.10*	
9	1.02 ± 0.13	2.46 ± 0.29	1.01 ± 0.10	4.45 ± 0.56****	p<0.
	1.00 ± 0.05	6.58 ± 1.48	1.03 ± 0.14	13.24 ± 2.64****	
	1.01 ± 0.09	24.51 ± 4.89	1.04 ± 0.15	20.16 ± 2.31**	
	1.07 ± 0.23	2.72 ± 0.77	1.03 ± 0.12	3.01 ± 0.27**	
5	1.03 ± 0.14	25.91 ± 6.52***	1.12 ± 0.32	18.56 ± 2.15****	

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GENE	Male Sham wk10 (normalised)	Male PMX wk10 FC±SD	Female Sham wk10 FC±SD (normalised)	Female PMX wk10 FC±SD	PMX Male vs Female
Inflammatory					
Ccl19	1.04 ± 0.19	1.50 ± 0.35	1.01 ± 0.09	0.92 ± 0.06	
Ccl2	1.02 ± 0.14	1.21 ± 0.34	1.04 ± 0.20	1.06 ± 0.16	
Ccr2	1.01 ± 0.13	1.18 ± 0.28	1.07 ± 0.25	1.12 ± 0.11	
Ccr7	1.02 ± 0.15	0.99 ± 0.21	1.03 ± 0.17	1.23 ± 0.10	
Cd14	1.01 ± 0.08	1.45 ± 0.44	1.05 ± 0.24	1.10 ± 0.09	
Il10	1.11 ± 0.38	1.69 ± 0.68	1.05 ± 0.21	1.60 ± 0.19	
Il15	1.05 ± 0.25	0.75 ± 0.13	1.01 ± 0.11	1.51 ± 0.23	p<0.05
Il1r1	1.01 ± 0.11	1.64 ± 0.18	1.02 ± 0.14	1.49 ± 0.15	
Il2	1.11 ± 0.37	0.99 ± 0.20	1.03 ± 0.16	1.48 ± 0.43	
Il4	1.01 ± 0.10	0.90 ± 0.31	1.02 ± 0.13	1.72 ± 0.21	
Il6	1.06 ± 0.28	0.88 ± 0.17	1.02 ± 0.15	1.30 ± 0.18	
Il6ra	1.06 ± 0.28	1.19 ± 0.20	1.01 ± 0.08	1.54 ± 0.21	
Nos2	1.02 ± 0.15	0.85 ± 0.09	1.03 ± 0.17	1.32 ± 0.14	p<0.05
Tnf	1.00 ± 0.02	1.45 ± 0.17	1.09 ± 0.30	1.30 ± 0.09	
Pain					
Bdkrb1	1.01 ± 0.09	2.48 ± 0.34 *	1.09 ± 0.32	1.57 ± 0.23	
Bdkrb2	1.00 ± 0.04	1.60 ± 0.35	1.08 ± 0.32	1.20 ± 0.10	
Cnr1	1.01 ± 0.11	0.89 ± 0.19	1.09 ± 0.33	1.10 ± 0.14	
Cnr2	1.02 ± 0.13	1.35 ± 0.20	1.02 ± 0.16	1.26 ± 0.17	
Gal	1.02 ± 0.13	1.15 ± 0.28	1.00 ± 0.05	1.21 ± 0.13	
Gdnf	1.01 ± 0.08	0.95 ± 0.20	1.06 ± 0.22	2.54 ± 0.30 *	p<0.01
Ngf	1.01 ± 0.10	1.76 ± 0.14 **	1.06 ± 0.26	1.56 ± 0.26	
Npy	1.03 ± 0.16	1.38 ± 0.48	1.08 ± 0.27	0.62 ± 0.08	
Nrtn	1.03 ± 0.19	1.07 ± 0.41	1.02 ± 0.15	6.71 ± 1.24 *	p<0.01
Ntf3	1.05 ± 0.24	1.03 ± 0.20	1.11 ± 0.37	1.92 ± 0.27	p<0.05
Ntf5	1.04 ± 0.21	0.33 ± 0.12 *	1.02 ± 0.14	2.89 ± 0.48	p<0.001
Penk	1.03 ± 0.18	1.19 ± 0.11	1.17 ± 0.46	1.16 ± 0.15	
Pspn	1.05 ± 0.23	1.19 ± 0.27	1.15 ± 0.44	0.40 ± 0.12	p<0.05
Tac1	1.01 ± 0.10	1.15 ± 0.44	1.06 ± 0.26	1.43 ± 0.15	
Tacr1	1.01 ± 0.09	2.17 ± 0.53	1.43 ± 0.85	1.68 ± 0.27	

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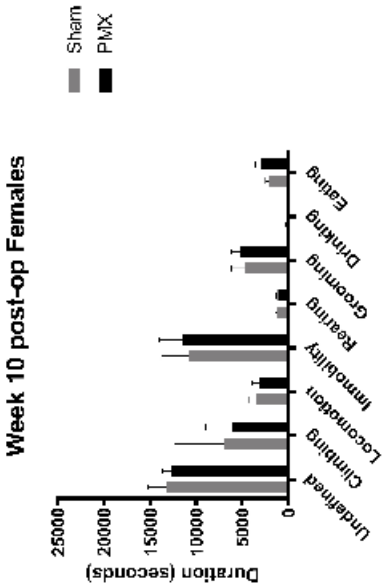
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PMX
Duration (seconds)
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20000
15000
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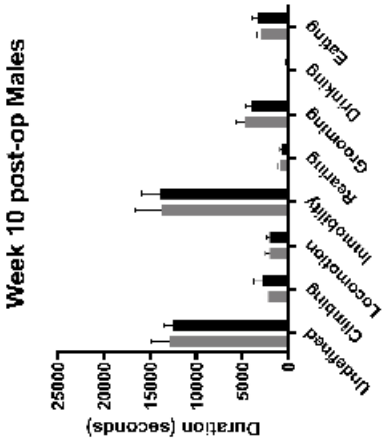
5000
0
Undefined
Climbing
Locomotion
Immobility
Rearing
Grooming
Drinking
Eating


Week 10 post-op Males
Duration (seconds)
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Undefined
Climbing
Locomotion
Immobility
Rearing
Grooming
Drinking
Eating

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A
Cartilage repair scores
Males
Females
Pineda Score
B
Naive
0 hours
Male
Female
8 weeks
C
Males
OARSI Score
Time after DMM
Sch after DMM
weeks
D
Females
OARSI Score
Time after DMM
Sch after DMM
weeks

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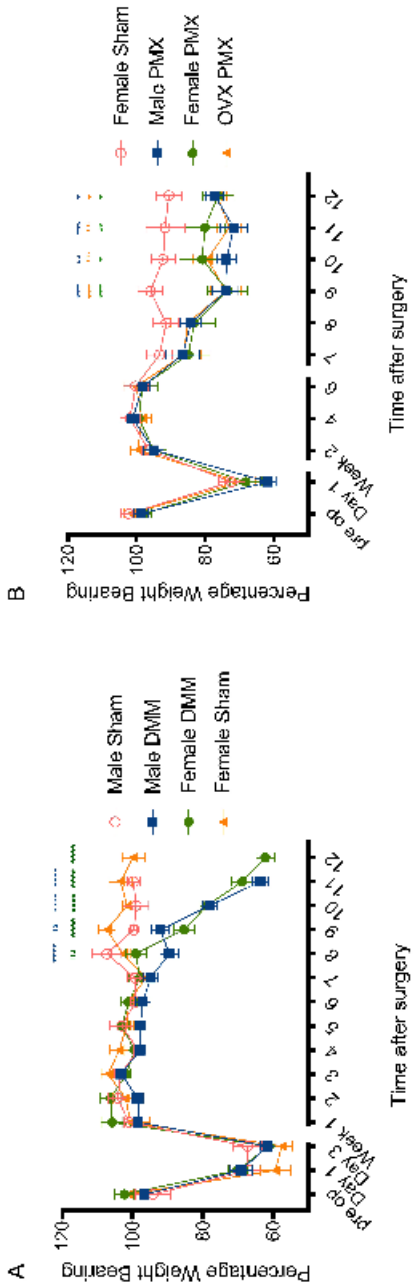
519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Female DMM
Female Sham
Pre op
Day 1
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Time after surgery
B
Percentage Weight Bearing
120
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Female Sham
Male PMX
Female PMX
OVX PMX
Pre op
Day 1
Week 2
3
4
5
6
7
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9
10
11
12
Time after surgery

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Supplementary Table 1: Genes examined on TaqMan Low-Density Array (TLDA) microfluidic cards

Gene with accession no.	Comments
Adamts1-Mm00477355_m1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1
Adamts4-Mm00556068_m1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4
Adamts5-Mm00478620_m1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)
Arg1-Mm00475988_m1	arginase, liver
Bdkrb1-Mm04207315_s1	bradykinin receptor, beta 1
Bdkrb2-Mm00437788_s1	bradykinin receptor, beta 2
Bmpr2-Mm00432134_m1	bone morphogenetic protein receptor, type II (serine/threonine kinase)
Ccl19-Mm00839967_g1	chemokine (C-C motif) ligand 19
Ccl2-Mm00441242_m1	chemokine (C-C motif) ligand 2
Ccr2-Mm01216173_m1	chemokine (C-C motif) receptor 2
Ccr7-Mm01301785_m1	chemokine (C-C motif) receptor 7
Cd14-Mm00438094_g1	CD14 antigen
Cd68-Mm03047343_m1	CD68 antigen
Cnr1-Mm00432621_s1	cannabinoid receptor 1 (brain)
Cnr2-Mm00438286_m1	cannabinoid receptor 2 (macrophage)
Ctgf-Mm01192932_g1	connective tissue growth factor
Fgf18-Mm00433286_m1	fibroblast growth factor 18
Fgf2-Mm00433287_m1	fibroblast growth factor 2
Fgfr1-Mm00438930_m1	fibroblast growth factor receptor 1
Fgfr2-Mm00438941_m1	fibroblast growth factor receptor 2
Fgfr3-Mm00433294_m1	fibroblast growth factor receptor 3
Fgfr4-Mm01341852_m1	fibroblast growth factor receptor 4
Gal-Mm00439056_m1	galanin
Gapdh-Mm99999915_g1	glyceraldehyde-3-phosphate dehydrogenase
Gdnf-Mm00599849_m1	glial cell line derived neurotrophic factor
Has1-Mm03048195_m1	hyaluronan synthase1
Il10-Mm00439616_m1	interleukin 10
Il15-Mm00434210_m1	interleukin 15
Il1a-Mm00439620_m1	interleukin 1 alpha
Il1b-Mm00434228_m1	interleukin 1 beta
Il1r1-Mm00434237_m1	interleukin 1 receptor, type I
Il2-Mm00434256_m1	interleukin 2
Il4-Mm00445260_m1	interleukin 4
Il6-Mm00446190_m1	interleukin 6
Il6ra-Mm00439653_m1	interleukin 6 receptor, alpha
Inhba-Mm00434339_m1	inhibin beta-A
Ltbp1-Mm00498234_m1	latent transforming growth factor beta binding protein 1
Ltbp2-Mm01307379_m1	latent transforming growth factor beta binding protein 2
Mmp13-Mm00439491_m1	matrix metallopeptidase 13

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Supplementary Table 2: Average raw CT values (± SEM) of genes associated with inflammation and pain in whole knees 10 weeks after PMX or sham surgery

Gene	M Sham Wk10 CT±SEM	M PMX Wk10 CT±SEM	F Sham Wk 10 CT±SEM	F PMX Wk 10 CT±SEM
Gapdh (control)	15.27 ± 0.15	15.55 ± 0.41	15.91 ± 0.30	15.92 ± 0.15
Inflammatory				
Ccl19	26.60 ± 0.14	26.53 ± 0.82	26.34 ± 0.26	26.48 ± 0.11
Ccl2	26.73 ± 0.08	27.06 ± 0.89	26.90 ± 0.08	26.88 ± 0.25
Ccr2	21.51 ± 0.10	21.67 ± 0.51	22.26 ± 0.13	22.13 ± 0.12
Ccr7	27.38 ± 0.10	27.86 ± 0.74	27.18 ± 0.10	26.91 ± 0.14
Cd14	25.96 ± 0.12	25.92 ± 0.62	26.22 ± 0.14	26.11 ± 0.12
Il10	29.11 ± 0.37	29.01 ± 0.74	29.19 ± 0.11	28.56 ± 0.21
Il15	23.93 ± 0.24	24.77 ± 0.72	25.01 ± 0.15	24.49 ± 0.20
Il1r1	24.91 ± 0.08	24.52 ± 0.50	25.24 ± 0.10	24.71 ± 0.23
Il2	31.73 ± 0.46	32.15 ± 0.60	32.77 ± 0.11	32.45 ± 0.49
Il4	28.07 ± 0.02	28.89 ± 0.86	29.22 ± 0.40	28.49 ± 0.10
Il6	27.85 ± 0.26	28.47 ± 0.74	28.60 ± 0.30	28.28 ± 0.22
Il6ra	24.22 ± 0.24	24.33 ± 0.57	24.63 ± 0.27	24.06 ± 0.08
Nos2	30.61 ± 0.32	31.17 ± 0.42	31.85 ± 0.24	31.49 ± 0.14
Tnf	25.66 ± 0.12	25.45 ± 0.44	25.92 ± 0.13	25.56 ± 0.09
Pain				
Bdkrb1	30.48 ± 0.09	29.52 ± 0.63	30.34 ± 0.12	29.75 ± 0.32
Bdkrb2	30.74 ± 0.16	30.48 ± 0.73	30.91 ± 0.17	30.68 ± 0.22
Cnr1	28.65 ± 0.16	29.27 ± 0.76	29.09 ± 0.19	29.01 ± 0.05
Cnr2	24.65 ± 0.10	24.59 ± 0.64	24.88 ± 0.09	24.61 ± 0.10
Gal	29.63 ± 0.25	30.32 ± 1.30	30.07 ± 0.34	29.85 ± 0.11
Gdnf	31.26 ± 0.22	31.86 ± 0.87	32.55 ± 0.64	31.25 ± 0.10
Ngf	29.74 ± 0.02	29.23 ± 0.49	29.56 ± 0.10	29.00 ± 0.36
Npy	28.03 ± 0.36	28.17 ± 0.75	27.61 ± 0.20	28.34 ± 0.12
Nrtn	27.52 ± 0.19	27.61 ± 0.42	29.86 ± 0.50	27.24 ± 0.36
Ntf3	27.28 ± 0.33	27.68 ± 0.74	28.04 ± 0.25	27.19 ± 0.30
Ntf5	29.14 ± 0.28	31.45 ± 0.99	31.72 ± 0.50	30.29 ± 0.27
Penk	24.96 ± 0.25	25.03 ± 0.51	26.00 ± 0.26	25.85 ± 0.15
Pspn	30.85 ± 0.19	30.69 ± 0.57	29.18 ± 0.55	30.89 ± 0.62
Tac1	29.61 ± 0.08	30.08 ± 0.85	29.89 ± 0.30	29.42 ± 0.22
Tacr1	31.61 ± 0.22	30.94 ± 0.62	31.68 ± 0.61	31.01 ± 0.28

*n=5 for PMX, n=3 for sham surgery.